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Establishment of an in vitro micropropagation protocol for *Boscia senegalensis* (Pers.) Lam. ex Poir.

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Abstract: This report describes in vitro micropropagation of *Boscia senegalensis*, so-called famine foods, that helped the people in Darfur and Kordofan, Sudan survive during the 1984–1985 famine. Four types of explants prepared from green mature zygotic embryos were cultured on Murashige and Skoog (MS) medium augmented with 1–5 mg/L 6-benzyladenine (BA). The highest number of shoots per explant (14.3±0.9) was achieved on MS medium supplemented with 3 mg/L BA, while the highest shoot length [(3.5±0.4) cm] was obtained with 1 mg/L BA. The shoot cluster, when subcultured to its same medium, significantly increased the rate of shoot multiplication by the end of the third subculture. The maximum mean number of shoots per explant (86.5±3.6) was produced after three multiplication cycles on 3 mg/L BA-supplemented medium. In vitro induced shoots were excised and rooted on half strength MS medium fortified with 0.25 mg/L indole-3-butyric acid (IBA) to obtain complete plantlets. *B. senegalensis*-regenerated plantlets obtained in vitro for the first time, were hardened and 95% survived under greenhouse conditions.

Key words: *Boscia senegalensis*, Famine food, Mature embryo, Organogenesis
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1 Introduction

Boscia senegalensis (Pers.) Lam. ex. Poir. is an evergreen shrub reaching up to 7 m in height (Neuwinger, 1996). It is native to the Sahel and Sahara savannas stretching from Mauritania, Senegal, Mali, Niger, and Nigeria to Cameroon and across Africa to Egypt, Sudan, Ethiopia, Somalia, and Kenya (Neuwinger, 1996; Orwa *et al*., 2009). The Sahel area, particularly the Sudanese ecological zone, within the semiarid and arid regions is characterized by extremely high temperatures and low rainfall (Salih *et al*., 1991). *B. senegalensis* is very well adapted to this drought region with some of the hottest and driest location conditions ever faced by higher plant life (Salih *et al*., 1991; Dicko *et al*., 2005). It tolerates shade temperatures as high as 45 °C and survives with 100 mm annual rainfall, but 250 mm is sufficient for vigorous growth (Dicko *et al*., 2001; Orwa *et al*., 2009). In addition to this hard climate condition, the shrub occupied soil of poor quality, stony slopes, sand dunes, and cracking-clay plains, which are rocky, desiccated, and even fire-scorched sites (Orwa *et al*., 2009).

The importance of *B. senegalensis* for the rural agro-economy in Africa has been illustrated by several reports, making it a plant of high value for both humans and animals (Neuwinger, 1996; Dicko *et al*., 2001; 2005). Its seed flour commonly replaces

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sorghum, millet, or lentils when making porridge (Dicko *et al*., 2005). During the 1984–1985 famine in Kordofan and Darfur, western Sudan, the international aid efforts did not reach all affected areas. However, mortality due to malnutrition in these areas was not as high as predicted due to the reliance of the people on so-called famine foods (de Waal, 1989). Salih *et al*. (1991) reported that 71% of starved people in northern Darfur and 38% in northern Kordofan consumed *B. senegalensis*, which was locally named Mukheit, during the famine compared with 56% of respondents in southern Darfur and 11% in southern Kordofan. de Waal (1989) raised the percentage of people in northern Darfur to 94%. People in many areas of the Sahel avoid grain purchases almost completely due to lack of income, relying instead on *B. senegalensis* fruits (Muller and Almedom, 2008; Pedersen and Benjaminsen, 2008).

The presence of several carbohydrate hydrolases in the leaves of *B. senegalensis* could justify their traditional use for sweet cereal-based foods (Dicko *et al*., 2001; 2005). The plant is traditionally used as medicines for many infection diseases like colic, bilharzia, eczema, and for male sexual weakness (Neuwinger, 1996). In addition, the fruits proved to be used as a coagulating agent to clarify turbid water (Neuwinger, 1996). Moreover, *B. senegalensis* contributes to nutrient cycling in sands, preventing soil erosion and degradation, as a sand dunes stabilizer, windbreaker, and for other useful purposes in harsh sites where people need every last bit of help in the struggle to survive (Neuwinger, 1996).

Although *B. senegalensis* is a very important multipurpose shrub, there are no effective methods for propagation in nursery or direct planting in the field mainly because the seeds rapidly lose their viability and their embryos are killed when seed water contents become below 20%–30% and frequently attacked by seed borers due to its high sugar content (Berjak and Pammenter, 2004; Pritchard *et al.*, 2004; Yu *et al.*, 2008). Recruitment is thus likely favoured by availability of such sites, making the species dependent on large tree species in arid savannah. Therefore, haphazard removal of that species represents a threat to *B. senegalensis*. Under ideal conditions, *B. senegalensis* seeds germinate rapidly compared to other arid-zone species, but their germination rates are low and growth rates are slow as reported by Rinaudo *et al.* (2002). The low rate of germination is common in *Boscia* species, which it reported to be 27.8% for *Boscia albitrunca* (Alias *et al*., 2003). The slow growth rate of this deep-rooted species is likely attributable to extensive investment in establishing and maintaining a deep taproot system, prior to above-ground growth (Canadell *et al*., 1996).

The main prerequisite for the ex-situ conservation, establishment of plantations, and tree improvement, is the production of a large number of plants of superior selected germplasms (Siddique and Anis, 2009). There is a need, therefore, to exploit other alternative techniques, like in vitro tissue culture technologies which offer fascinating methods for large-scale production of plants in shorter duration devoid of seasonal constraints (Raghavan, 2003). Embryo culture, for many recalcitrant species, is an important milestone for mass micropropagation of plants from a small number of original seeds and a convenient initial source for the establishment of shoot cultures because of their juvenile nature (Chaturvedi *et al*., 2004). Therefore, the objective of the present study was to investigate the responses of *B. senegalensis* tissues in vitro to growth regulators at a range of concentrations in order to identify optimum conditions for adventitious regeneration of shoots from embryo. To our knowledge, this is the first report of in vitro study for *B. senegalensis*.

2 Materials and methods

2.1 Plant materials

Fully-grown green fruits (Fig. 1b) were collected from adult *B. senegalensis* shrubs (Fig. 1a) at Al-Rawakeeb Research Station, West Omdurman, Sudan, during the first two weeks of February 2009, and were used throughout the experiment.

2.2 Surface sterilization

The fruits of *B. senegalensis* were washed thoroughly in running tap water to remove any soil attached to the fruits' surfaces. Aseptically, the fruits were surface-sterilized in 70% (v/v) ethanol for 3 min, followed by 15% (v/v) Clorox commercial bleach solution [4.5%–5.0% (v/v) sodium hypochlorite] for 15 min, and then rinsed five times with autoclaved distilled water. After sterilization and prior to embryo

isolation, the seeds were kept in autoclaved distilled water to facilitate zygotic embryo excision. Embryos (10 mm×5 mm) were aseptically dissected without endosperms by tearing out the fleshy pulp and the seed peeled from the sterilized fruits using sharp surgical blades and tweezers (Fig. 1c). Then, the embryos were immersed and rinsed five times in autoclaved distilled water for removal of the growth inhibitors and to hasten the germination process. Surface moisture was removed with a sterilized filter paper prior to culture.

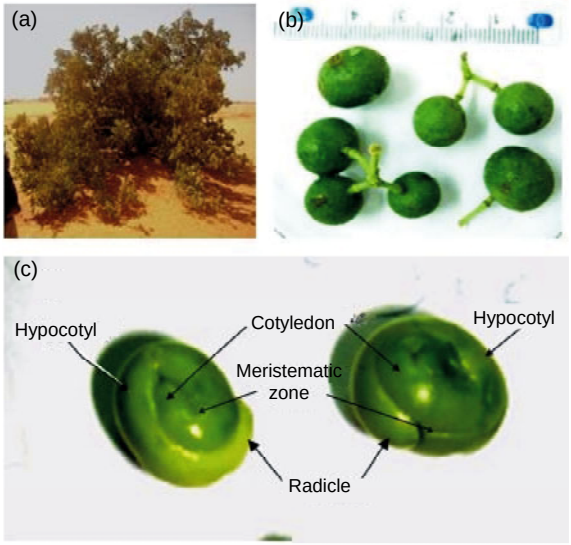

Fig. 1 Plant materials and embryos
(a) Adult plants of *B. senegalensis* growing on sand dunes in Al-Rawakeeb area; (b) Green mature fruits collected from the area; (c) Explant: green mature zygotic embryo after extracts from the fruits in rounded form before sterilization and germination

2.3 Explant preparation

The extracted embryos were utilized directly in this study as explants for shoot induction or to prepare another three types of explants. Therefore, there were four types of explants in the experiment. Embryo explant (EM), which is the initially extracted embryo, included the intact embryonic axis (shoot meristem+ hypocotyl) with cotyledons (Fig. 2a). The other three types of embryo explants were prepared using 7–10 d old seedlings derived from in vitro germinated embryos (Fig. 2b) on half strength (half mineral and organic contents) hormone-free MS (Murashige and Skoog, 1962) medium in 15-ml tubes (20 cm×3 cm) under dark conditions, as follows: (1) type CN: embryo with excised half of the cotyledons and hypocotyls-radicle axis (Fig. 2c); (2) type HC: hypocotyl segments prepared by removing the cotyledons from the top and radicle of embryo axis from the bottom (Fig. 2d); (3) type RA: the root axis (Fig. 2e). All types of explants were implanted in the multiplication media on 30-ml bottles (9 cm×2 cm).

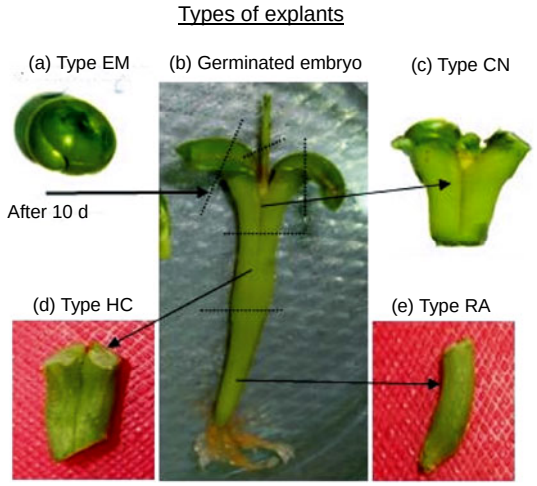

Fig. 2 Preparation of *B. senegalensis* embryo explants
(a) Embryo extracted from seed in rounded form represents embryo explant type EM. (b) Embryo after 10 d germinates to "Y" form on half strength MS medium under dark conditions. After that, the germinated embryo was used to prepare the other three explants. (c) Embryo explant type CN (cotyledonary node) was prepared by removing half of the cotyledons from the top of the embryo and the hypocotyl-radicle axis from the bottom. (d) Embryo explant type HC (hypocotyl) was prepared by removing the cotyledons and the shoot primordia from the top and radicle from the bottom. (e) Embryo explant type RA (root axis) was prepared by removing the cotyledons, the shoot primordia, and the major parts of hypocotyls from the top

2.4 Shoot induction and multiplication

MS media supplemented with 0, 1, 2, 3, and 5 mg/L 6-benzyladenine (BA) alone or in combination with α-naphthalene acetic acid (NAA) in different concentrations (0.0, 0.1, 0.5, and 1.0 mg/L) were used to investigate its effects as multiplication media. The BA was supplemented alone and at a wide range of concentrations in an attempt to evaluate and stimulate clonal shoot multiplication and shoot development. The maximum number of shoots and shoot length were recorded after four weeks of culture on multiplication media.

To evaluate the effect of subculture, each embryo explant produced shoots and was transferred to the same fresh multiplication medium, after harvesting the induced shoots ≥1.5 cm in length, and was separated carefully by using a small knife under sterilized conditions to avoid any damage. The multiplication rate was tested up to three passages and total number of shoots and shoot length were recorded at each passage of subculturing. Multiplication rates were calculated as the difference in mean shoot number between the mean numbers of shoots derived before and after subculture from one culture at the end of each passage. The averages of number of shoots and multiplication rate were also determined for the three subculture passages per medium.

2.5 In vitro rooting of shoots

For rooting of in vitro induced shoots, half strength MS media supplemented with indole-3-butyric acid (IBA) or NAA at 0.00, 0.25, 0.50, 1.00 or 1.50 mg/L, were used as rooting media. Shoots of 1.0–1.5 cm were harvested from multiple shoots and then cultured in rooting media. Data were recorded on percentage of rooting and the number and length of roots/shoots after four weeks of culture.

2.6 Media and culture conditions

Semi-solid MS basal medium was prepared and used in all cultures. The medium was adjusted to the desired pH 5.8 using HCl or NaOH. A gelling of 7 g/L agar was added, and heated until the solution was clear and dispensed into the culture vessels before autoclaving. The medium was sterilized in a validated autoclave at 15 psi (1 psi=6.895 kPa) at 121 °C for 15 min. The culture room had a constant temperature of (25±2) °C, under cool fluorescent light of about 5000 lx and a photoperiod of 16-h light and 8-h dark.

2.7 Acclimatization of plantlets

Rooted plantlets of about 6 cm in length (4 cm long shoot, 2 cm long root) were washed thoroughly in running tap water, and transplanted into an autoclaved plastic pots (5 cm×10 cm) filled with mixture of sand:silt and soil (3:1, v/v) under artificial diffuse light (16 h/8 h photoperiod) conditions, and then were covered with polyethylene bags and kept in the laboratory. After two weeks, the pots were transferred to a plastic greenhouse under 30 °C with relative humidity 50%–60% and were watered daily. The regenerated plantlets were acclimatized for four to eight weeks and then successfully transferred to the soil under greenhouse condition.

2.8 Statistical analysis

The experiment was conducted using a completely randomized design with ten replications. Two experiments were conducted separately for each type of explant. The mean of replications was used for statistical analysis. For each of the four explant types, the MS medium was supplemented with five concentrations. Mean separation was performed using Duncan's multiple range test at 0.05 probability level.

3 Results and discussion

3.1 Explant preparation

In this study, mature zygotic embryos of *B. senegalensis* (Fig. 2a) were either cultured on MS basal media without growth regulators to prepare other three types of explants, or cultured on MS basal media with different concentrations of BA to inspect a suitable medium for in vitro micropropagation. Choice of the explant is critical for efficient plant regeneration (Ebrahimie *et al*., 2006). The zygotic embryo is an important milestone for mass micropropagation of plants because of their juvenile nature (Chaturvedi *et al*., 2004).

After a week of culture on hormone-free MS medium, the embryos spread in Y shape with cotyledons (Fig. 2b). A preliminary study (data not shown) indicated that, BA, an adenine-type cytokinin, in all concentrations investigated, induces multiple buds and adventitious shoots on embryo explants. The use of *N*-phenyl-*N'*-1,2,3-thiadiazol-5-yl-urea (thidiazuron, TDZ), a substituted phenylurea cytokinin, and 2,4-dichlorophenoxyacetic acid (2,4-D) was stimulatory to callus formation on embryos in all media and concentrations, whereas NAA was found to be stimulatory to shoot growth and root elongation; it appeared to not generate multiple shoots. This was the proof of using NAA in the multiplication medium. Moreover, the use of TDZ might not be able to induce multiple shoots or organogenesis because of its auxin- and cytokinin-like activities, which might be disrupting the delicate balance necessary for shoot bud

formation. TDZ might be impinging upon the endogenous auxins by their modified biosynthesis and/or their protection in vivo (Mehta *et al*., 2004). Thiem (2003) reported that callus growth on explant usually interferes with the propagation process.

3.2 Effects of BA and explant types on multiple shoot formation

After four weeks of culture, all explant types failed to form multiple shoots on a growth regulator-free MS medium but remained green. Zygotic embryos (EM) responded morphogenetically and germinate to seedlings. However, on the medium containing cytokinins, various numbers of adventitious shoots per explant were sprouted as shoot clusters at all BA concentrations, but the frequency of shoot formation was influenced by explant types and BA concentrations. The superiority of BA has also been reported for number of leguminous species (Bopana and Saxena, 2009).

The dose of cytokinin is known to be critical for the induction of multiple shoots. Among the BA concentrations, BA at 3 mg/L was found to be significantly more effective in producing a greater number of shoots per explant. It showed the maximum number of shoots produced (14.8) by EM explant (Table 1; Fig. 3a). The high response of type EM to shoot formation can be attributed to several factors. Initially, in contrast to the other explant types, the cells of embryo explant type EM were 10 d younger at the time of exposure to BA concentrations (Fig. 2b). Also, it is merely a combination of all the three previous explants types. Type EM explant, unlike other types, generates shoots in two pathways: shoot induction from pre-existing meristem and the direct shoot organogenesis pathway from de novo meristematic cells.

The cotyledonary node (CN), ranked second in the explant types, produced 9.3±0.6 shoots per explant (Fig. 3b, Table 1). The 1 mg/L BA produced significantly higher shoot length [(3.5±0.4) cm], as was observed in the experiment on CN explants. Similar observations of plant regeneration using cotyledon explants with intact cotyledonary nodes were reported among legumes (Ebrahimie *et al*., 2007). Evidently, the presence of cotyledons was essential for realizing maximum shoot production potential. If the shoots are primarily arising from the existing buds of the embryonic axis, the shoot number should not be affected when half of the cotyledons are removed (Polisetty *et al*., 1997). Apical meristematic cells in the cotyledonary node are sites for hormone synthesis and occasionally exhibit different needs of plant growth regulators for regeneration in comparison to other tissues (Shimizu-Sato *et al*., 2009). As well, the excision of the radical might stimulate multiple shoot production. *B. senegalensis* is a drought-resistant plant with a high antioxidant activity. These kinds of plants increase endogenous cytokinin to high levels in response to drought stress and in this way become tolerant to drought (Ebrahimie *et al*., 2007). That, the internal cytokinins act as an antioxidant defence system and decrease the damaging effects of drought (Yordanov *et al*., 2000). Moreover, it has been observed that the rises in endogenous cytokinin content and its production require the presence of NAA or BA in the medium (Ebrahimie *et al*., 2007).

The HC and RA explants did not contain a meristematic zone (Figs. 2d and 2e), so they form shoots through direct organogenesis. Within two

Table 1 Effect of BA on shoot induction of *B. senegalensis* explants after four weeks without subculture

Explant type	c_{BA} (mg/L)	Number of shoots/explant[1]	Mean length of shoot (cm)[2]
CN	0	0.0 ± 0.0^{g}	0.0 ± 0.0^{h}
	1	3.3 ± 0.8^{efg}	3.5 ± 0.4^{a}
	2	8.3 ± 0.9^{bc}	1.6 ± 0.1^{cd}
	3	9.3 ± 0.6^{b}	1.7 ± 0.1^{c}
	5	7.2 ± 0.8^{bcd}	0.6 ± 0.1^{fg}
HC	0	0.0 ± 0.0^{g}	0.0 ± 0.0^{h}
	1	1.2 ± 0.3^{fg}	1.8 ± 0.3^{c}
	2	3.3 ± 0.4^{efg}	1.0 ± 0.2^{ef}
	3	5.5 ± 0.5^{cde}	1.7 ± 0.1^{c}
	5	3.2 ± 0.7^{efg}	0.5 ± 0.1^{fg}
RA	0	0.0 ± 0.0^{g}	0.0 ± 0.0^{h}
	1	1.3 ± 0.3^{fg}	0.3 ± 0.1^{g}
	2	2.2 ± 0.3^{efg}	0.5 ± 0.0^{fg}
	3	3.0 ± 0.4^{efg}	0.7 ± 0.1^{fg}
	5	4.1 ± 0.5^{def}	0.8 ± 0.1^{efg}
EM	0	0.0 ± 0.0^{g}	0.0 ± 0.0^{h}
	1	3.2 ± 0.9^{efg}	2.5 ± 0.5^{b}
	2	10.0 ± 0.7^{b}	1.2 ± 0.1^{de}
	3	14.3 ± 0.9^{a}	0.6 ± 0.0^{fg}
	5	10.3 ± 0.8^{b}	0.4 ± 0.1^{g}

c_{BA}: 6-benzyladenine (BA) concentration. [1] Only shoots with ≥1.5 cm length; [2] Shoots produced per treatment. Values are expressed as mean±standard error (SE). Means followed by the same superscript letter within the same column are not significantly different (P=0.05) using Duncan's multiple range test

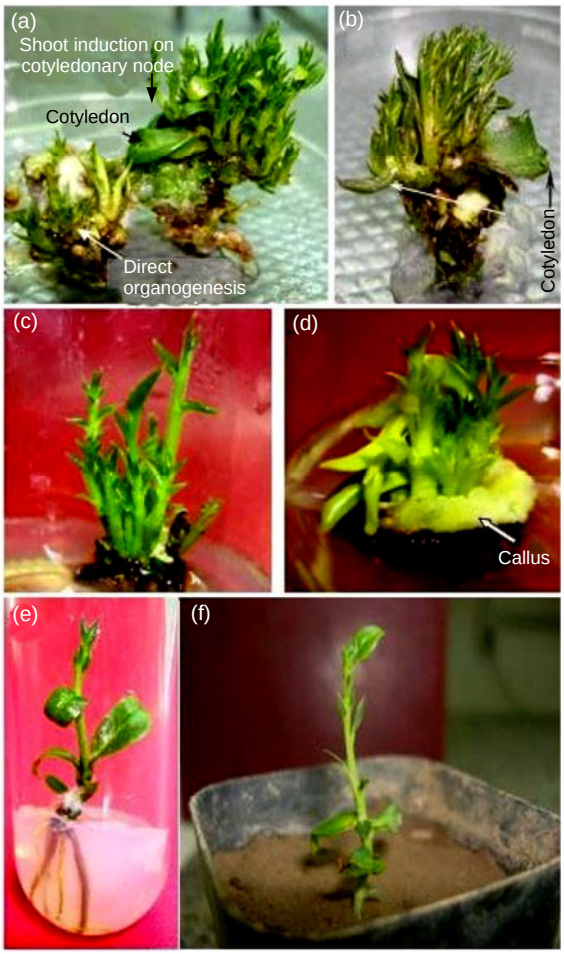

Fig. 3 Induction and regeneration of different in vitro morphogenesis pathways from different embryo explant types after inoculation on 3 mg/L BA medium for four weeks of culture

(a) Morphologic alterations in type EM (embryo); (b) Multiple shoot induction on type CN (cotyledonary node); (c) Direct organogenesis on type HC (hypocotyl); (d) Direct organogenesis on type RA (radicle) with presence of callus; (e) Plants in vitro rooted on half strength MS containing 0.25 mg/L IBA after four weeks of culture and hardened for another four weeks; (f) Acclimatized micropropagated plants at the end of the third week after ex vitro growth on soil under laboratory conditions

weeks, both explants started to swell and induced budding on the surface and bottom of explant that was laying on the medium. The organogenic tissues were then rapidly proliferated to direct organogenesis shoots after three weeks of culture on the same medium. The maximum number of organogenic shoots was established after four weeks of culture on the same medium (Figs. 3c and 3d). The HC explant produced a maximum number of shoots (5.5±0.5). However, unlike other explants, the RA explant required the high concentration of 5 mg/L BA to produce the maximum number of shoots (4.1±0.5) (Fig. 3d). The lower induction of shoots in HC and RA explants, when compared to EM and CN explants, even at 2 mg/L BA (Table 1), indicated the superiority of meristems in the shoot formation over unmeristematic zones. The direct induction of shoot buds on internodes in the presence of only a cytokinin without the need of an exogenous auxin is not an often encountered phenomenon since the internodes do not have preformed meristems. This result may be attributed to the high intrinsic auxin levels in the internodes of this plant; therefore, by supplementing them with high concentrations of BA, it is possible to balance both of the growth regulators so that the explant becomes competent for organogenesis (Christianson and Warnick, 1985).

The intermediate concentrations of BA (2–3 mg/L) appeared to favour bud formation over the low concentration (1 mg/L) and shoot growth over the high concentration (5 mg/L). The shoots developed on 5 mg/L BA, with a length of (0.4±0.1) cm, did not elongate further (Table 1). Reduction in the number of shoots in the concentration higher than optimal level has also been reported for several woody plants (Rai *et al*., 2009). The number of shoots calculated for BA at 5 mg/L as shown in Table 1, did not explain the precise number of shoots induced but only the ones that can be counted, e.g., more than 1.5 cm in length. So, the mean number of shoots shown by the BA at 2 mg/L was magnified as this concentration produced larger microshoots than BA at 5 mg/L. Cytokinins, particularly BA, commonly stimulate shoot proliferation and inhibit elongation (Albarello *et al*., 2006). Furthermore, at higher cytokinin levels, the RA explant produced excessive calluses and failed to improve the efficiency of shoot multiplication (Fig. 3d). Thiem (2003) reported that callus growth on explants usually interferes with the shoot formation process. The effectiveness of BA to induce multiple shoots was also reported in other woody species such as *Pterocarpus marsupium* (Anis *et al*., 2005), *Cleome spinosa* (Albarello *et al*., 2006), *Acacia senegal* (Khalafalla and Daffalla, 2008), *Balanites aegyptiaca* (Siddique and Anis, 2009), and *Psidium guajava* (Rai *et al*., 2009).

3.3 Effect of subculture on shoot multiplication

The effect of subculture passage was evaluated on shoot multiplication in an MS medium supplemented with different levels (1, 2, 3 and 5 mg/L) of BA after transfer to the same medium. Shoot multiplication was observed on explants after removing the shoots taller than 1.5 cm. Shoots shorter than 1.5 cm were kept in the same medium to undergo further proliferation and elongation. Subculture significantly increased multiple shoots' induction and direct organogenesis. The maximum mean number (86.5±3.6) of shoots after three multiplication cycles was produced on the medium supplemented with 3 mg/L BA (Fig. 4). The caulogenic potential of shoots decreased after the third multiplication cycle with the greatest number of shoots of (25.8±2.3) with 1 mg/L BA. The average number of shoots of all three subculture passages was calculated to be 50±0.5.

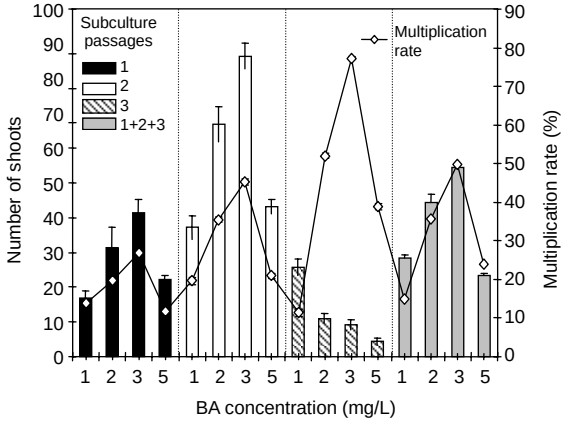

Fig. 4 Effects of subculture passages on number of shoots and multiplication rate of *B. senegalensis* obtained from BA (1, 2, 3, or 5 mg/L) on the same medium
Bars represent mean numbers of shoots (±SE) and line represents the mean of multiplication rates

An important factor affecting the efficiency of a micropropagation system is the rate of multiplication, which can be calculated as the ratio of shoot number at the end of the subculture to the initial number of shoots (Mendes *et al*., 1999). The formations of adventitious buds of *B. senegalensis* on all explants at both the initiation and the multiplication stages were observed. In the case of prolonged culture on the initiation media, the number of new buds continued to increase into the second subculture phase and decrease into the third one. The effect of BA in the initiation medium became evident in the first subculture. The multiplication rate of *B. senegalensis* was occasionally very high (up to 50 shoots) depending on the BA concentration in the medium (Fig. 4). At a continuous multiplication of *B. senegalensis* cultures on low-BA (1 mg/L) media, the multiplication rate began to decrease, starting from the second subculture to the third subculture. An increase in the concentration of cytokinin led to an increase in the multiplication rate and to a decrease in the shoot number. Although 1 mg/L BA resulted in good proliferation, showing the highest number of shoots in last passage and formation of long shoots, during a long-term culture on media with BA, the multiplication rate decreased [(11.3±0.9)%]. Generally, the caulogenic potential of shoots decreased after the third multiplication cycle. Likewise, tissues of *Cleome spinosa* (Albarello *et al*., 2006) and *Balanites aegyptiaca* (Siddique and Anis, 2009) were reported to lose their proliferation capacities after five subcultures on BA. According to the report of Albarello *et al*. (2006), a decrease in the propagation rate after several subcultures may require elimination of cytokinin for one or two passages in order to improve the shoot multiplication.

3.4 Effect of additional auxin on shoot induction

For further proliferation, the optimal concentration of BA (3 mg/L) was tested with different concentrations of NAA (0.1, 0.5, and 1.0 mg/L) using only EM explants. Inclusion of NAA in combination with BA in the culture medium was not effective in enhancing shoot proliferation (Table 2). The MS medium supplemented with BA (3 mg/L) and NAA (0.1 mg/L) produced less shoots per explant (10.8±0.5) compared to that containing BA alone (14.4±0.4). Low NAA concentrations appeared to be more effective than high NAA concentrations. Higher concentrations of NAA (1.0 mg/L) were not beneficial, as it resulted in callus formation at the base of explant and reduced bud induction and multiplication. In consistency with this result, Vengadesan *et al.* (2002) have reported that when CN explants of *Acacia sinuata* were cultured on an MS medium containing a combination of BA and auxins (NAA, IBA, and IAA), the shoots were reduced in number but in turn produced basal callus. In the present work, the use of cytokinins also induced shoot production through direct organogenesis from hypocotyl and radicle explants. An

important advantage of direct organogenesis is the potential for maintaining genomic stability of regenerated plants with low incidences of somaclonal variation and chromosomal abnormalities (Ebrahimie *et al*., 2006), whereas regeneration via an intermediate callus phase increases the possibility of somaclonal variations (Tang and Guo, 2001). However, using DNA molecular marker techniques is preferred to ensure that the in vitro regenerants are true to the parent type (Martins *et al*., 2004).

Table 2 Effects of various concentrations of NAA on multiple shoot induction from the *B. senegalensis* embryo after four weeks of culture

NAA concentration (mg/L)	Number of shoots/explant
0.0	14.4±0.4[a]
0.1	10.8±0.5[b]
0.5	7.4±0.5[c]
1.0	6.8±1.1[c]

BA at 3 mg/L. Values are expressed as mean±SE of at least nine replicates. Means followed by the same superscript letter within the same column are not significantly different (P=0.05) using Duncan's multiple range test

3.5 In vitro rooting of microshoots

For any micropropagation protocol, successful rooting of microshoots is a prerequisite to facilitate their establishments in soil. Only microshoots with 1.5 cm length can be considered useable for a rooting experiment. Shoots shorter than 1.5 cm are not easily harvestable from an impenetrable shoot cluster (Figs. 3a and 3b) and were likely to be injured when trying to separate with use of blades. However, harvesting was carried out after four weeks (of culture on multiplication media) or eight weeks (from first subculture). Rooting occurred sporadically one to two weeks when shoots were transferred to hormone-free medium or to media containing IBA or NAA (Table 3). Using this procedure, 100% of shoots were successfully rooted after four weeks of culturing with either 0.25 mg/L IBA- or 1.0 mg/L NAA-containing media. The occurrence of root formation on auxin-free medium (88%) may be due to the availability of endogenous auxin in in vitro raised shoots. In vitro roots appeared to be straight, not branched, thick and strong (Fig. 3e). Table 3 showed that IBA, in all its applications, appears better than NAA in terms of minimum concentration needed for rooting frequency, average number of roots (1.6±0.4), and average root length [(1.7±0.3) cm]. IBA has been shown to be very effective in root induction in various species of tropical trees such as *Azadirachta indica* (Chaturvedi *et al*., 2004), *Albizia odoratissima* (Rajeswari and Paliwal, 2008), *Psidium guajava* (Rai *et al*., 2009), and *Balanites aegyptiaca* (Siddique and Anis, 2009).

Table 3 Effects of IBA and NAA on root induction of in vitro raised *B. senegalensis* shoots after eight weeks of culture

Auxin	Rooting (%)	Number of roots/shoot	Root length (cm)
Control	88.0	1.8±0.5	1.0±0.2
IBA			
0.25 mg/L	100.0	2.4±0.3	2.3±0.4
0.50 mg/L	87.5	1.3±0.3	1.6±0.2
1.00 mg/L	62.5	1.1±0.4	1.5±0.4
1.50 mg/L	87.0	1.6±0.4	1.2±0.3
Average		1.6±0.4	1.7±0.3
NAA			
0.25 mg/L	50.0	1.0±0.3	0.4±0.1
0.50 mg/L	62.0	1.1±0.4	0.3±0.1
1.00 mg/L	100.0	2.1±0.2	0.5±0.1
1.50 mg/L	63.0	1.5±0.6	0.7±0.1
Average		1.2±0.4	0.5±0.1

Values are expressed as mean±SE of at least eight replicates

Variation in rooting response was observed among the regenerated shoots taken from different subcultures. Shoots excised from initial cultures had a good rooting potential while the shoots taken from later subcultures exhibited poor rooting response (data not shown). Rooted plantlets were transferred to plastic pots containing sterile soil (Fig. 3f). Within four to five weeks, new leaves appeared, which indicated root growth.

4 Conclusions

These results describe the development of two different plant regeneration pathways from four different explants of *B. senegalensis*. Four types of explants prepared from green mature zygotic embryo were cultured on MS media augmented with 1–5 mg/L BA. The highest number of shoots per explant (14.3±0.9) was achieved on the MS medium supplemented with 3 mg/L BA, while the highest shoot length [(3.5±0.4) cm] was obtained with

1 mg/L BA. Other three different tissue types were used as the source materials for the *B. senegalensis* (Mukheit) in vitro propagation systems. The most significant observation of these studies was the significantly greater expression of regenerative potential in the intact embryo cultures as compared to the other three different types of explants. In vitro culture of *Boscia* will be beneficial for efficient genetic conservation by organ culture, the production of useful secondary materials by cell culture, and basic cellular level research for the elucidation of its unique biological character. Shoot organogenesis is directly induced from embryo of *B. senegalensis* without an intervening callus. This drastically reduces the duration of shoot regeneration in vitro. It is important to point out that the present study describes for first time in vitro regeneration of *B. senegalensis*.

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